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The bacterial degradation of nicotine and related compounds.

(Not included: certain additional information obtained on the second product. A copy of a paper on this will be received soon. It is being sent to the Journal of Biological Chemistry.)

The third oxidative product:

Since our last report in October 1958 in which very cursory information on the third oxidative step in the bacterial oxidation of nicotine was given, we have pursued this phase of the problem further. The initial investigations along this line were directed towards the further elucidation of the conditions necessary for formation of the third oxidative product. These investigations showed the following conditions optimal for the conversion of nicotine, 6 hydroxynicotine, or 6 hydroxypseudooxynicotine to third product with the uptake of 1.5, 1.0 and 0.5 μm of oxygen per μm of substrate respectively.

a) The fraction of the crude enzyme precipitating between 40 and 60 per cent ammonium sulfate saturation contains the enzyme system necessary for this conversion.

b) The pH optimum for formation of third product from second product lies between 7.5 and 8.0.

c) The product is formed in the presence of methylene blue or brilliant cresyl blue and the 40-60 ammonium sulfate fraction. In the case of the former dye the oxidation proceeds slowly past the third product stage and in the case of the latter stops at the third step.

d) The optimum concentration of BCB was found to be 0.08 μm of the dye under conditions where enzyme concentration was not limiting.

With the conditions for the formation of the third product established, we next attempted a large scale isolation of the compound. This was done in the Warburg apparatus using 800 mg of 6-hydroxynicotine as the starting material. The oxidation was followed in a Warburg vessel containing an aliquot of the main reaction mixture which was shaken in the Warburg in an Erlenmeyer flask. The progress of the formation of the product was also followed spectrophotometrically using diluted aliquots from the main flask. The decrease in the absorbance at 290 mμ (6HN) and the increase at 360 mμ (third product) were measured. When oxidation ceased in the Warburg vessel and the 360 absorbance remained stationary, the reaction was presumed to be complete.

The reaction mixture was deproteinized by heating at 65° C for 15 minutes and the precipitated protein removed by centrifugation. The clear supernate was brought to pH 1.0 with concentrated HCl and put on a Dow 50 ion exchange column in the hydrogen form. After washing with water and 0.3 M NH_4OH the compound was eluted with 0.9 M NH_4OH. All of the 20 ml fractions showing appreciable 360 mμ absorbance were combined and taken to dryness in vacuo.

The yellow-brown residue was extracted with amyl alcohol from which, upon concentration and slow cooling, needles separated out. After five recrystallizations from butanol 200 mg. of faintly pink colored needle-shaped crystals were obtained. The isolated compound has the following physical and chemical properties:

a) It forms a yellowish solution in water, alcohol, acetic acid and mineral acids. It is relatively insoluble in ether.

b) It adsorbs strongly in the UV having 2 maxima at 290 and 360 mμ. The 360/290 ratio of the compound is 5.15.

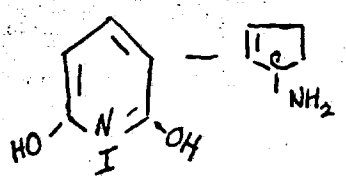
c) The uncorrected melting point on a block is 258-260 C.

d) The compound is homogeneous when chromatographed on paper and gives a violet spot when sprayed with acid $FeCl_3$. The compound gives a blue color characteristic of dihydroxy pyridines when tested with the Folin-Ciocalteu phenol reagent.

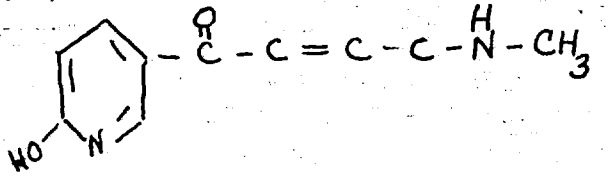
e) The elemental analysis calculated for $C_{10}H_{12}N_2O_2$ was:

Calc.		Found	
C	62.5	C	61.22
H	6.25	H	6.31
N	14.55	N	14.22

From the above data two possible basic structures may be postulated for third product, a dihydroxy-N-methyl myosmine (I) or a compound containing a double bond in the open side chain (II).



I



At present the evidence is more in favor of I (the second hydroxyl may be in the 2,4 or 5 position) because of the typical diphenol type reactions obtained with ferric chloride and the Folin reagent. The compound, whatever its structure, is apparently new to nicotine chemistry since nothing resembling its properties has been found in the literature.

A strange situation exists in the case of the isolated compound however. Under none of the conditions so far employed have we been able to obtain further oxidation of the purified compound. These conditions include tests of enzymes, crude and fractionated, resting cells, and dried cells, all with and without methylene blue present.

We have several explanations for this anomalous behavior. First the product may be altered during the isolation procedure and second this product may not be on the main oxidative pathway. With respect to the second alternative it is possible that the BCB is acting as a poising agent that forces the formation of this product due to an alteration in the oxidation-reduction potential of the system. In favor of the second hypothesis is the finding that the material formed in the reaction mixture before chemical manipulations and the purified material are identical chromatographically and spectrophotometrically.

There is a third possibility which we have not as yet investigated. This involves the formation of the isolated product from some precursor which is not itself on the main pathway. Since the second product can exist in three forms dependent on pH it could well be that one of these forms gives rise to the isolated product and the other gives rise to the true third oxidation product.

We have carried out a few very preliminary studies on the formation of the fourth product and as yet are unable to state anything definite about its nature except to say that all UV absorbance disappears at this stage indicating that the pyridine ring has been ruptured. The fourth product has been found on paper chromatograms of reaction mixtures using a diazotizing reagent as the indicator.

In the next six months we hope to more fully characterize the isolated third product and to complete the identification of the substance. We expect to obtain a more concise picture of the biological significance of this compound and to elucidate its position in the oxidation of nicotine by this organism.

We will investigate the conditions necessary for the formation of the fourth product and will attempt to isolate and identify the compound in the near future.

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